Isolation of Arabidopsis Mutants With Enhanced Disease Susceptibility by Direct Screening

Jane Glazebrook, Elizabeth E. Rogers and Frederick M. Ausubel

Department of Genetics, Harvard Medical School and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

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ABSTRACT

To discover which components of plant defense responses make significant contributions to limiting pathogen attack, we screened a mutagenized population of *Arabidopsis thaliana* for individuals that exhibit increased susceptibility to the moderately virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326). The 12 enhanced disease susceptibility (*eds*) mutants isolated included alleles of two genes involved in phytoalexin biosynthesis (*pad2*, which had been identified previously, and *pad4*, which had not been identified previously), two alleles of the previously identified *npr1* gene, which affects expression of other defense genes, and alleles of seven previously unidentified genes of unknown function. The *npr1* mutations caused greatly reduced expression of the *PR1* gene in response to *Psm*ES4326 infection, but had little effect on expression of two other defense genes, *BGL2* and *PR5*, suggesting that *PR1* expression may be important for limiting growth of *Psm*ES4326. While direct screens for mutants with quantitative pathogen-susceptibility phenotypes have not been reported previously, our finding that mutants isolated in this way include those affected in known defense responses supports the notion that this type of screening strategy allows genetic dissection of the roles of various plant defense responses in disease resistance.

THE responses by which eukaryotic hosts defend themselves from pathogen attack have not been subjected to rigorous genetic analysis in any organism. This sort of analysis requires a tractable genetic system for the host, and well-characterized host-pathogen interactions. Clearly, vertebrates are unsuitable for such studies. A great deal of molecular and biochemical reasearch has been conducted using plant-pathogen systems, but many of the plant hosts are difficult subjects for detailed genetic studies due to large or polyploid genomes and long generation times. With the advent of well-characterized host-pathogen systems involving the model plant *Arabidopsis thaliana* as the host, genetic analysis of host defense responses has become feasible (Dangl 1993; Crute *et al.* 1994; Kunkel 1996). As described in more detail below, some progress has been made by screening for Arabidopsis mutants with defects in particular defense responses that have been identified biochemically (Cao *et al.* 1994; Glazebrook and Ausubel 1994; Delaney *et al.* 1995). However, to identify regulatory factors and other defense response components that are not already correlated with a known biochemical or molecular genetic response, it is necessary to screen directly for mutants that display enhanced susceptibility to pathogen attack.

If a general screen for enhanced susceptibility mutants were carried out in Arabidopsis, the mutants isolated in such a screen might include plants with defects in pathogen-inducible responses such as structural reinforcement of plant cell walls by lignification, synthesis of active oxygen species, synthesis of antimicrobial secondary metabolites (called phytoalexins), and accumulation of pathogenesis-related (PR) proteins. The PR proteins include chitinases, β-glucanases, and chitin-binding proteins (Kauffmann *et al.* 1987; Legrand *et al.* 1987; Ponstein *et al.* 1994), as well as others with unknown activities (Lamb *et al.* 1989; Dixon and Lamb 1990).

The facts that phytoalexins and many PR proteins inhibit pathogen growth *in vitro* suggest that they play a direct role in conferring resistance to particular pathogens. Indeed, phytoalexins are defined by their antimicrobial activity *in vitro* (Paxton 1981). In addition, chitinases, chitin-binding proteins, and β-1,3-glucanases inhibit the growth of various fungi (Schlumbaum *et al.* 1986; Mauch *et al.* 1988; Sela-Buurlage *et al.* 1993; Ponstein *et al.* 1994), the PR protein osmotin inhibits *Phytophthora infestans* (Woloshuk *et al.* 1991), and defensin-like proteins from radish have anti-fungal activity (Terras *et al.* 1992).

Additional evidence that phytoalexin and PR proteins play a direct role in conferring resistance is the observation that constitutive expression in transgenic plants of PR genes or constitutive expression of certain phytoalexin biosynthetic genes causes decreased disease susceptibility. For example, synthesis of a grape phytoalexin in transgenic tobacco led to increased resis-

Corresponding author: Jane Glazebrook, Center for Agricultural Biotechnology, 1105 Ag/Life Sciences Surge Building, College Park, MD 20742-3351. E-mail: glazebro@mbimail.umd.edu

tance to *Botrytis cinerea* (HAIN *et al.* 1993), constitutive expression of chitinase in tobacco caused increased resistance to *Rhizoctonia solani* (BROGLIE *et al.* 1991), expression of PR-1a in tobacco resulted in increased tolerance to *Peronospora tabacina* and *Phytophthora parasitica* (ALEXANDER *et al.* 1993), expression of osmotin at extremely high levels in potato conferred some resistance against *P. infestans* (but osmotin expression in tobacco failed to protect against *P. parasitica*) (LIU *et al.* 1994), coexpression of chitinases and glucanases in tobacco increased resistance to *Cercospora nicotianae* (ZHU *et al.* 1994), and expression of radish defensins in tobacco enhanced resistance to *Alternaria longipes* (TERRAS *et al.* 1995). In related studies, increasing hydrogen peroxide levels by expression of glucose oxidase in potato enhanced resistance to *Erwinia carotovora* and *P. infestans* (WU *et al.* 1995).

Isolation of plant mutants with defects in particular defense responses would allow the roles of these responses in combatting particular pathogens to be examined by studying the effects of their loss on plant-pathogen interactions. Such studies would complement transgenic plant studies, such as those described above, in which the roles of particular responses are examined by studying the effects of their constitutive expression, or expression in heterologous hosts, on plant-pathogen interactions. *A priori*, it seems that a genetic approach to study the plant defense response might be complicated by functional redundancy arising from the plethora of responses elicited by pathogen attack. However, the phenotypes of some recently isolated Arabidopsis mutants suggest that a genetic dissection of the defense response is not only feasible but may uncover previously unknown defense mechanisms.

Arabidopsis *pad* mutants are deficient in the synthesis of camalexin, the only phytoalexin that has been found in significant quantities in Arabidopsis (TSUJI *et al.* 1992; GLAZEBROOK and AUSUBEL 1994). Two of the *pad* mutants (*pad1 and pad2*), but not a third (*pad3*), allowed significantly more growth of the virulent bacterial pathogen, *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326), than wild-type plants did (GLAZEBROOK and AUSUBEL 1994).

Another Arabidopsis mutant, *npr1*, was isolated in a screen for mutants affected in systemic acquired resistance (SAR) (CAO *et al.* 1994). SAR is a phenomenon wherein infection of a plant with a necrotizing pathogen leads to accumulation of PR proteins in the uninfected leaves, which concomitantly become resistant to a variety of normally virulent pathogens (ENYEDI *et al.* 1992; MALAMY and KLESSIG 1992). Salicylic acid plays an important role as a signaling compound in SAR (GAFFNEY *et al.* 1993), and treatment of plants with salicylic acid leads to PR protein accumulation and pathogen resistance (ENYEDI *et al.* 1992; MALAMY and KLESSIG 1992). The *npr1* mutant failed to express the PR genes *PR1*, *BGL2*, and *PR5* in response to salicylic acid and failed to become systemically resistant in response to infection by a necrotizing pathogen (CAO *et al.* 1994). Even in the absence of SAR-inducing stimuli, the *npr1* mutant allowed more growth of *Psm* ES4326 than wild-type plants did (CAO *et al.* 1994). The *nim1* mutant displays similar phenotypes to *npr1* mutants (DELANEY *et al.* 1995), suggesting that *npr1* and *nim1* may be allelic. These results show that some genes involved in SAR are also involved in limiting pathogen growth in the abscence of SAR, suggesting that a screen for enhanced disease susceptibility mutants might yield mutants with defects in SAR.

In this work, we describe the results of a screen for Arabidopsis mutants with enhanced disease susceptibility (*eds* mutants). In previous work, we observed that while strain *Psm*ES4326 causes severe disease symtoms characterized by water-soaking and chlorosis of the infected tissue when plants are infected at a high dose, much weaker symptoms, limited to isolated chlorotic spots, result from infection at a lower dose. Importantly, *pad1* and *pad2* mutants infected at the lower dose display much more severe symptoms than wild-type plants (GLAZEBROOK and AUSUBEL 1994). Based on this observation, we screened plants grown from mutagenized seed for those that displayed more severe symptoms than wild-type plants after infection with a low dose of *Psm*ES4326. The resulting collection of *eds* mutants included *pad* mutants, *npr1* mutants, and mutants with defects in seven other genes not previously identified by mutation.

MATERIALS AND METHODS

Bacteria, plants, growth conditions, and genetic crosses: *P. syringae* pv. *maculicola* ES4326 (*Psm*ES4326) has been described (DONG *et al.* 1991). *Psm*ES4326 *degP-46* contains a transposon Tn*phoA* insertion in a gene whose product shares 42% identity and 59% similiarity to the periplasmic serine protease encoded by the *Escherichia coli degP* gene (L. STEVENS and F. AUSUBEL, unpublished data). Bacteria were cultured in King's B medium (protease peptone, 10 mg/ml; glycerol 15 mg/ml; K_2HPO_4, 1.5 mg/ml; $MgSO_4$, 4 mM pH7.0) supplemented with 100 μg/ml streptomycin at 28°. *A. thaliana* ecotype Columbia (referred to as "Arabidopsis-Col-0" for simplicity) was grown in Metromix 2000 (W. R. Grace) soil, either in a climate-controlled greenhouse (20 ± 2°, relative humidity 70 ± 5%) on a 16-hr light/8-hr dark cycle or in a Conviron growth chamber (20 ± 2°, 90% relative humidity) on a 12-hr light/dark cycle under 125 μEinsteins of fluorescent illumination. The screen for mutants displaying enhanced sensitivity to *Psm*ES4326 was conducted in the growth chamber, while the screen for mutants displaying enhanced sensitivity to *Psm*ES4326 *degP-46* was conducted in the greenhouse. Experiments involving determination of bacterial symptoms and growth were carried out in the growth chamber. Other experiments were conducted in the greenhouse. Plants were infected with suspensions of bacterial cells in 10 mM $MgSO_4$ by using a 1-ml syringe (without a needle) to force the suspension through the stomata by pressing the syringe against the abaxial side of the leaves. Genetic crosses were performed by dissecting immature flowers of the pollen recipient before

TABLE 1

Sequences of oligonucleotide primers used for generation of probes

Gene	Sense primer	Antisense primer
BGL2	CTACAGAGATGGTGTCA	AGCTGAAGTAAGGGTAG
PR1	GTAGGTGCTCTTGTTCTTCCC	CACATAATTCCCACGAGGATC
PR5	CACATTCTCTTCCTCGTGTTC	TAGTTAGCTCCGGTACAAGTG
UBQ5	GTGGTGCTAAGAAGAGGAAGA	TCAAGCTTCAACTCCTTCTTT

anther dehiscence and applying pollen from the pollen donor to the recipient pistil.

Preparation of mutagenized seed: Ecotype Columbia plants that were homozygous for the *fah1-2* mutation (CHAPPLE *et al.* 1992) were used for mutagenesis. Seeds were soaked in a 0.2% (v/v) solution of ethylmethane sulfonate (EMS) in water for 16 hr with gentle agitation. They were washed extensively with water, and sowed on soil. M2 seed was collected from these plants, and divided into 12 pools each representing approximately 400 M1 plants. Approximately 5% of the M2 plants grown from this seed displayed morphological alterations.

***Psm*ES4326 growth assay:** Plants grown in a growth chamber were infected with *Psm*ES4326 at a dose of 10^3 cfu/cm^2 leaf area (determined in practice as a dilution equivalent to OD_{600} = 0.0002) After 3 days, growth was assayed by excising a sample consisting of two 0.28 or 0.18 cm^2 disks from each infected leaf using a cork borer, grinding the sample in 10 mM $MgSO_4$ using a plastic pestle, and plating appropriate dilutions on King's B medium containing streptomycin. Data are reported as means and standard deviations of the log(cfu/cm^2) of six to eight replicates.

Camalexin determination: Plants were infected with *Psm*ES4326 at a dose of 10^5 cfu/cm^2 leaf area, and 36–48 hr later camalexin was extracted from infected tissue and visualized on thin layer chromatography plates under long-wave ultra-violet illumination as described previously (GLAZEBROOK and AUSUBEL 1994)

Treatment of seedlings with salicylic acid: Seeds were germinated and grown for 13 days on Murashige and Skoog medium (Gibco BRL) solidified with 0.8% agar (MS medium) under 50 μEinsteins continuous fluorescent illumination at 20°. They were then transferred either to fresh MS medium, or to MS medium containing 0.5 mM sodium salicylate. After a further 6 days of growth, seedlings were harvested and stored at −80° before preparation of RNA.

RNA blot analysis: Total RNA was purified from Arabidopsis leaves as described (REUBER and AUSUBEL 1996). Samples (5 μg) were separated on formaldehyde-agarose gels (AUSUBEL *et al.* 1995), transferred to GeneScreen hybridization membrane, hybridized to various probes (described below) and washed according to the instructions of the supplier (New England Nuclear, Boston, MA).

Probes were prepared by amplification of appropriate sequences using the polymerase chain reaction (PCR), purification of the products on agarose gels, and labeling of single stranded probes by PCR using these purifed products, antisense primers and α-^{32}P-dCTP. Sequences of the oligonucleotide primers are shown in Table 1. For *BGL2*, a fragment extending between nucleotides 1133 and 1419 was aplified from plasmid pATBG12 (DONG *et al.* 1991). For *PR1* and *PR5*, fragments extending between nucleotides 61 and 480, and 65 and 740, respectively, were amplified from cDNA clones of *PR1* and *PR5* provided by Dr. ERIC WARD, of Ciba-Geigy Co. (UKNES *et al.* 1992). For *UBQ5*, a fragment extending between nucleotides 334 and 585 that was kindly provided by Dr. JEAN GREENBERG (University of Colorado at Boulder) was amplified. Blots were analysed using a phosphoimager (Molecular Dynamics). The data shown in Figure 4 were obtained by volume integration of the signals in the bands hybridizing to the *BGL2*, *PR5*, or *PR1* probes, and then normalizing these values to the values obtained by volume integration of the signals in the bands hybridizing to the *UBQ5* probe, to compensate for lane-to-lane variations in the amounts of RNA.

RESULTS

Isolation of mutants that allow enhanced growth of *P. syringae*: We performed two different screens in an effort to identify Arabidopsis mutants that display enhanced sensitivity to *P. syringae*. For both screens, we used plants grown from EMS mutagenized M2 seed of the Columbia ecotype that were homozygous for the *fah1-2* allele. The *fah1-2* line was chosen because *fah1-2* plants lack sinapoyl malate in the leaf epidermis, causing them to appear red, rather than blue, under ultraviolet illumination (CHAPPLE *et al.* 1992). This makes *fah1-2* a convenient marker in genetic crosses. Responses to *Psm*ES4326 are unaffected by the *fah1-2* mutation (J. GLAZEBROOK and F. M. AUSUBEL, unpublished data).

In the first screen, two leaves of each M2 plant were infected with strain *Psm*ES4326 at a dose of 10^3 cells/cm^2 leaf area. Wild-type plants infected with this concentration of *Psm*ES4326 show very weak symptoms manifested as small chlorotic spots 3 days after infection. Plants that displayed more extensive chlorosis were considered to be candidate enhanced disease susceptibility (*eds*) mutants. The chlorotic appearance of one such *eds* mutant is shown in Figure 1. Fifty-three *eds* candidates were identified among ~5000 plants screened. These plants were grown to maturity and M3 seed was collected from them.

In the second screen, we sought to identify plant mutants that compensated for the pathogenicity defect of a *Psm*ES4326 mutant, strain *Psm*ES4326 *degP-46*. This strain, which contains transposon Tn*phoA* inserted into the *degP* gene, grows to a final density in infected leaves that is 100-fold lower than that of *Psm*ES4326 and is more sensitive to oxidative stress imposed *in vitro* than *Psm*ES4326 is (L. STEVENS and F. AUSUBEL, unpublished data). We hypothesized that the reduction in virulence might be a consequence of a reduced ability to withstand an oxidative stress imposed by the plant host during an oxidative burst and that some of the plant mutants obtained by screening mutagenized plants for enhanced sensitivity to strain *Psm*ES4326 *degP-46* might

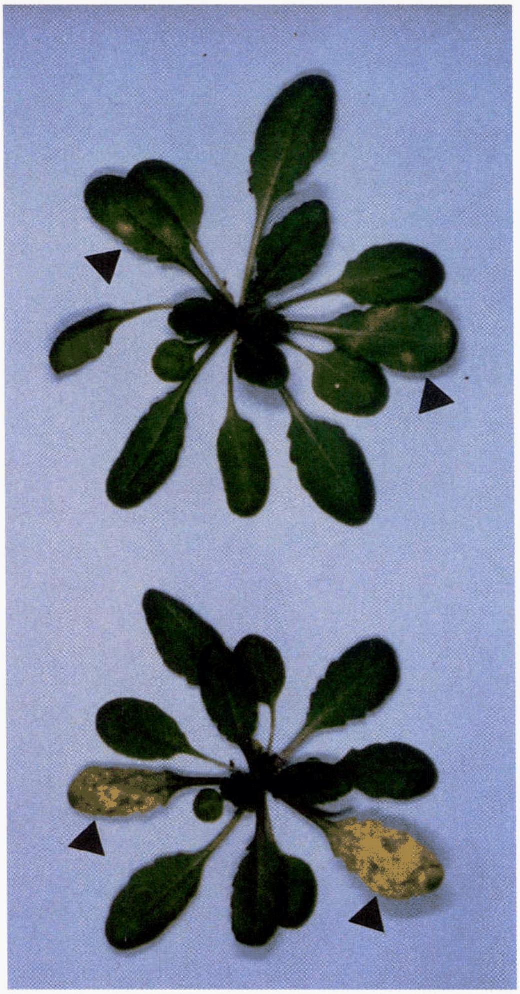

FIGURE 1.—*Psm*ES4326 causes more severe symptoms in *eds* mutant plants than it does in wild-type plants. Arrowheads indicate leaves that were infected with *Psm*ES4326 at a dose of 10^3 cfu/cm^2 leaf area. Plants were photographed 72 hr after infection. The top plant is the *fah1-2* parental genotype, the bottom plant is homozygous for *fah1-2* and the *eds-4* mutation.

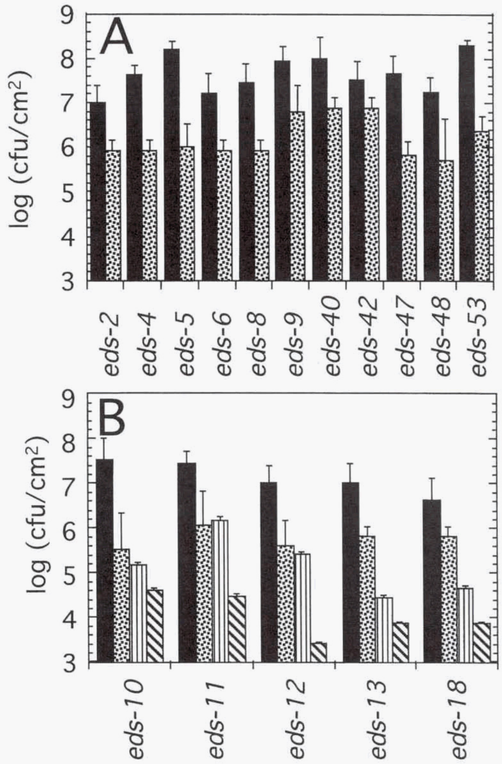

FIGURE 2.—Densities of *Psm* strains in wild-type and *eds* mutant plants 72 hr after inoculation. Plants were infected with *Psm*ES4326 or *Psm*ES4326 *degP-46* at a dose of 10^3 cells/cm^2 leaf area. After 72 hr, infected leaves were excised and bacterial density was determined as log(cfu/cm^2 leaf area). Each bar represents the mean and standard deviation of values from six replicate samples. Bacterial densities varied significantly among experiments, so only values obtained in the same experiment can be compared. Therefore, wild-type values obtained in the same experiment are shown for each *eds* mutant. (A) *eds* mutant plants isolated by screening with strain *Psm*ES4326. Solid bars, *Psm*ES4326 in the indicated *eds* mutant (M3 generation) plants and speckled bars, *Psm*ES4326 in wild-type (*fah1-2* parent line) plants. (B) *eds* mutant plants isolated by screening with *Psm*ES4326 *degP-46*. Solid bars, *Psm*ES4326 in the indicated *eds* mutant (M3 generation) plants; speckled bars, *Psm*ES4326 in wild-type (*fah1-2* parent line) plants; vertically striped bars, *Psm*ES4326 *degP-46* in *eds* mutant plants; and diagonally striped bars, *Psm*ES4326 *degP-46* in wild-type (*fah1-2* parent line) plants.

have defects in an oxidative defense mechanism. Such oxidative-defense mutations would be expected to have a stronger effect on growth of strain *Psm*ES4326 *degP-46* than on growth of *Psm*ES4326. In practice, we screened M2 plants (a separate M2 population from the first screen) by infecting two leaves of each plant with strain *Psm*ES4326 *degP-46* at a concentration of 10^4 cells/cm^2 leaf area. Plants that displayed more extensive chlorosis than wild-type plants 3 days after infection were considered to be candidate mutants. Eighty-three such plants were identified among ~7500 plants screened.

Candidate mutants identified in both screens were retested in the M3 generation using the *Psm*ES4326 growth assay described in MATERIALS AND METHODS. Based on this analysis, 16 of the 136 candidate mutants from the two screens were found to allow significantly more growth of *Psm*ES4326 than wild-type plants and were therefore judged to be *eds* mutants (Figure 2). The growth of strain *Psm*ES4326 *degP-46* in the candidate mutants isolated in the second screen was tested similarly. All of the mutants that allowed increased growth of strain *Psm*ES4326 *degP-46* also allowed a similar increase in growth of *Psm*ES4326 (Figure 2B). We concluded, therefore, that no mutants that specifically compensated for the growth defect of strain *Psm*ES4326 *degP-46* had been isolated. Since there does not appear to be any substantive difference between the mutants isolated in the two screens, they will be discussed as one group.

Two of the *eds* mutants are phytoalexin deficient: It is known that some classes of Arabidopsis mutants with defects in synthesis of the Arabidopsis phytoalexin, camalexin, allow enhanced growth of *Psm*ES4326 (GLAZEBROOK and AUSUBEL 1994). Therefore, we tested whether the *eds* mutants synthesized wild-type levels of camalexin in response to infection by *Psm*ES4326. Based on visual examination of camalexin fluorescence in a thin-layer chromatography (TLC) assay (see MATERIALS AND METHODS), we found that *eds-9* and *eds-47* synthesized significantly less camalexin than wild-type plants after infection by *Psm*ES4326. Mutants *eds-9* and *eds-47* were crossed to wild-type plants and to the previously isolated camalexin-deficient mutants *pad1-1, pad2-1,* and *pad3-1.* Camalexin was assayed in the F_1 plants after infection with *Psm*ES4326. In all genotypes, camalexin levels were not significantly different from levels in wild-type plants, except for the *eds-47* × *pad2-1* F_1 plants, which exhibited greatly reduced camalexin levels (10–30% of wild-type levels, as judged by visual examination of camalexin fluorescence). The results of these complementation tests show that: both *eds-9* and *eds-47* are recessive mutations; *eds-9* complements *pad1-1, pad2-1,* and *pad3-1,* so it is an allele of a new *pad* gene *(pad4);* and *eds-47* fails to complement *pad2,* so it is a *pad2* allele *(pad2-2).* Unexpectedly, The *eds-47* mutant did not show the *fah1-2* fluorescence phenotype. Despite this, we doubt that it is simply a *pad2-1* contaminant of the mutagenized seed, because the veins of the *eds-47* mutant were yellow, a phenotype that the *pad2-1* mutant lacks. Mutants *eds-9* and *eds-47* will be described in more detail in a separate publication concerning these and other newly isolated phytoalexin-deficient mutants (J. GLAZEBROOK and F. M. AUSUBEL, unpublished data).

Two of the eds mutations are *npr1* alleles: The only other Arabidopsis mutant that is known to allow increased growth of *Psm*ES4326 is *npr1-1* (CAO *et al.* 1994). Another *npr1-1* phenotype is failure to induce the defense genes *PR1, PR5,* and *BGL2* in response to salicylic acid. To test whether any of the *eds* mutants might be similar to *npr1-1,* we used RNA blot hybridization to compare the levels of the *PR1* transcript in wild-type and *eds* mutant seedlings grown in the presence or absence of salicylic acid. As shown in Figure 3, in wild-type plants and all of the *eds* mutants except *eds-5* and *eds-53,* the levels of *PR1* mRNA were much higher in plants grown in the presence of salicylic acid than they were in plants grown without salicylic acid. In *eds-5* and *eds-53,* however, *PR1* mRNA levels were unaffected by salicylic acid, demonstrating that the phenotypes of *eds5* and *eds-53* were similar to that of *npr1-1.*

Before conducting complementation tests to determine whether *eds-5* and *eds-53* were alleles of *NPR1,* or alleles of another gene(s), we first determined whether *eds-5* and *eds-53* were recessive or dominant. The *Psm*ES4326 growth assay was conducted on wild-type, mutant, and F_1 hybrid plants. The results [as log(cfu/cm^2 leaf area)], for *eds-5* were: Col-0, 5.72 ± 0.93; *eds-5,* 7.65 ± 0.23; and F_1 *eds-5* xCol-0, 5.73 ± 0.43; and for *eds-53* were: Col-0, 6.39 ± 0.32; *eds-53,* 8.29 ± 0.12; and F_1 *eds53* xCol-0, 7.02 ± 0.41. We concluded that *eds-5* and *eds-53* were recessive to the wild-type allele(s). The *npr1-1* mutation was already known to be recessive to *NPR1* (CAO *et al.* 1994).

We then crossed *npr1-1, eds-5,* and *eds-53* together in pairwise combinations. *Psm*ES4326 growth was assayed in these F_1 plants and in wild-type and mutant control plants. The results in Table 2 show that *eds-5* and *eds-53* both fail to complement *npr1-1* and are therefore alleles of *NPR1.* Mutants *npr1-2* and *npr1-3* were isolated from different pools of M2 seed, so it is likely that *npr1-2* and *npr1-3* are different alleles. Since the *Psm*ES4326 growth phenotypes of the F_1 plants used in this experiment were indistinguishable from those of the parental lines, it was desirable to ascertain whether the F_1 plants were true cross-progeny. It is certain that the F_1 *eds-5* × *npr1-1* plants were cross and not *npr1-1* self-progeny, because they did not display the early-bolting phenotype of *npr1-1.* The F_1 *npr1-1* × *eds53* plants must have been cross-progeny, rather than *eds-53* self-progeny, because they did not display the fluorescence phenotype of *fah1-2* homozygotes (*eds-53* is homozygous for *fah1-2*). We cannot be certain that F_1 *eds-53* × *eds-5* plants were true cross-progeny, but since *npr1-1* and *eds-5* are allelic, and *npr1-1* and *eds-53* are allelic, *eds-5* and *eds-53* must also be allelic. Consequently, we will now refer to *eds-5* as *npr1-2,* and *eds-53* as *npr1-3.*

Induction of PR1 gene expression in response to infection is impaired in *npr1* mutants: The observations that *npr1* mutants display enhanced pathogen sensitivity and fail to induce defense gene expression in response to salicylic acid treatment (CAO *et al.* 1994) suggested that the reason for the enhanced pathogen sensitivity might be failure to induce defense gene expression in response to pathogen attack. To test this hypothesis, we infected wild-type, *npr1-2,* and *npr1-3* plants with *Psm*ES4326 at a dose of 10^4 cells/cm^2 leaf area, and monitored defense gene expression in the infected tissue using RNA blot hybridization. As shown in Figure 4, we found that both *npr1-2* and *nrp1-3* did not differ significantly from wild-type plants with respect to induction of the defense genes *BGL2* and *PR5.* However, expression of *PR1* was induced to much lower levels in both of these *npr1* mutants than it was in wild-type plants. The expression of *PR1* in the *npr1* mutants was clearly induced relative to levels in uninfected tissue, in contrast to the case of salicylic acid treatment, in which *PR1* mRNA levels in *npr1* plants were unaffected by salicylic acid treatment.

The *eds* mutants represent 10 complementation groups: Complementation testing was carried out to determine how many complementation groups are defined by the *eds* mutants. Some of the *eds* mutants were excluded from this analysis for the following reasons. We assumed that *eds-9* and *eds-47* were not allelic with any of the other *eds* mutations, because unlike the other *eds* mutants, *eds-9* and *eds-47* are phytoalexin deficient. Similarly *npr1-2* and *npr1-3* are not likely to be allelic with any of the other *eds* mutants, because in all the other

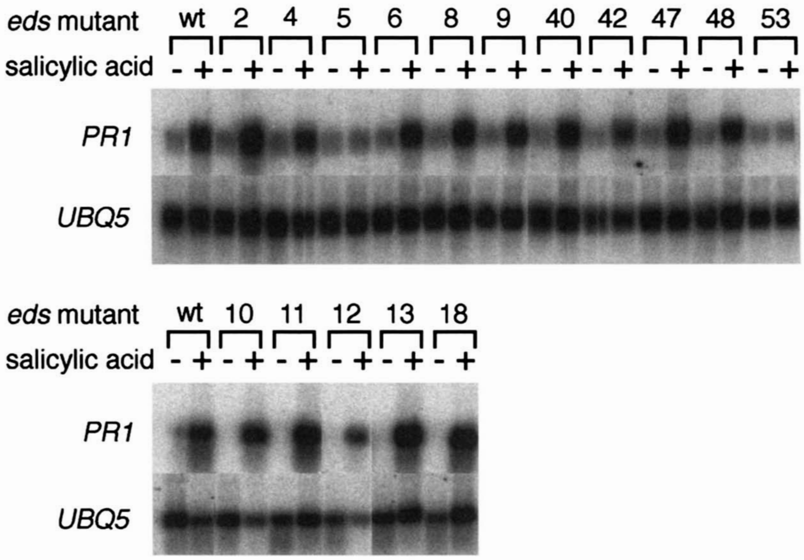

FIGURE 3.—Expression of the *PR1* gene in response to salicylic acid. RNA samples were hybridized with a *UBQ5* probe as a loading control. After stripping of the *UBQ5* probe, samples were hybridized with a *PR1* probe. Samples in the top row were analyzed in a separate experiment from samples shown in the bottom row, so the two rows cannot be directly compared. Lanes that were not relevant to this work were removed from the images by computer editing. wt, *fah1-2* parent line; − and +, plants grown without or with 0.5 mM salicylic acid, respectively.

eds mutants, *PR1* expression is induced in response to salicylic acid. *eds-10* plants were much smaller, and less green, than wild-type plants, and we were unable to identify plants with an *eds* phenotype among a F_2 population resulting from an *eds-10* × Col-0 cross, implying that the *eds-10* phenotype is caused by mutations at more than one locus. The phenotypes of *eds-40* and *eds-42* were inconsistently reproducible.

The remaining eight *eds* mutants were crossed to each other and to Col-0 in pairwise combinations. The resulting F_1 plants were compared with each parent, and with Col-0 plants, using the *Psm*ES4326 growth assay and visual inspection of infected leaves. For each F_1 genotype, three to eight plants were assayed. Mutations were judged to be complementing when *Psm*ES4326 growth and severity of chlorotic symptoms in F_1 plants resembled those of Col-0 plants. As shown in Table 3, all of these eight *eds* mutations were recessive, *eds-6* and *eds-8* failed to complement each other, but all of the other *eds* mutations complemented each other. Mutants *eds-6* and *eds-8* were isolated from the same pool of M2 seed; therefore, *eds-6* and *eds-8* could be the same mutation. In total, the *eds* mutations define 10 genes

TABLE 2

The mutations *eds-5* and *eds-53* are alleles of *NPR1*

Plant genotype	*Psm* ES4326 density 72 h after inoculation [log(cfu/cm^2)[a]
Col-0	5.79 ± 0.62
npr1	8.03 ± 0.36
eds-5	8.00 ± 0.40
eds-53	7.86 ± 0.50
F_1 *eds-5* × *npr1*[b]	7.82 ± 0.46
F_1 *npr1* × *eds-53*[b]	8.06 ± 0.41
F_1 *eds-53* × *eds-5*[b]	7.96 ± 0.36

[a] Values are means ± standard deviations of six replicates.
[b] F_1 *A* × *B* indicates pollen from plants of genotype *A* was used to fertilize plants of genotype *B*.

involved in limiting growth of *Psm*ES4326, including two genes involved in phytoalexin synthesis, *NPR1* and seven other *EDS* genes. The mutations in these seven *EDS* genes are renamed as follows: *eds-2, eds2-1*; *eds-4, eds3-1*; *eds-6, eds4-1*; *eds-8, eds4-2*; *eds-11, eds5-1*; *eds-13, eds6-1*; *eds-18, eds7-1*; and *eds-48, eds8-1*. The name *eds1* was not used, because Dr. JANE PARKER has named another mutation that affects disease susceptibility *eds1* (JANE PARKER, personal communication).

The phenotypes of most of the *eds* mutants result from mutations in single nuclear genes: F_2 plants derived from crosses between *eds* mutants and wild-type plants were assayed to determine the segregation patterns of the phenotypes of the *eds* mutants. Wild-type parent, *eds* parent, and F_2 progeny plants were infected with *Psm*ES4326 at a dose of 10^3 cfu/cm^2 leaf area, and chlorotic symptoms were visually inspected after 3 days. F_2 plants displaying symptoms similar to wild-type plants were scored as Eds$^+$, and those displaying symptoms similar to *eds* plants were scored as Eds$^-$. Table 4 shows that the results obtained for *npr1-2, npr1-3, eds2-1, eds3-1, eds4-1, eds4-2, eds5-1, eds6-1*, and *eds8-1* are consistent with the hypothesis that their Eds phenotypes segregate in a 3:1 ratio. The Pad$^-$ phenotype of *pad4* (*eds-9*) segregated 57 Pad$^+$:20 Pad$^-$ (χ^2 = 0.04 0.8 < P < 0.9 for 3:1 segregation) among the F_2 progeny of a *pad4* × Col-0 cross. It is therefore likely that all of these mutations are recessive alleles of single nuclear genes. The segregation of the Pad$^-$ phenotype of *pad2-2* was not examined. Segregation of the Eds phenotypes of the *eds7-1*, *eds-40*, and *eds-42* mutants was not examined because the Eds$^-$ phenotypes of these mutants are weaker than than those of the other *eds* mutants, and therefore we consider them less interesting. The *eds-12* mutant appears to have a complicated genotype, as the M3 *eds-12* line segregates some phytoalexin-deficient plants. It will be necessary to separate the putative *pad* and *eds* mutations in this line before examining their segregation patterns.

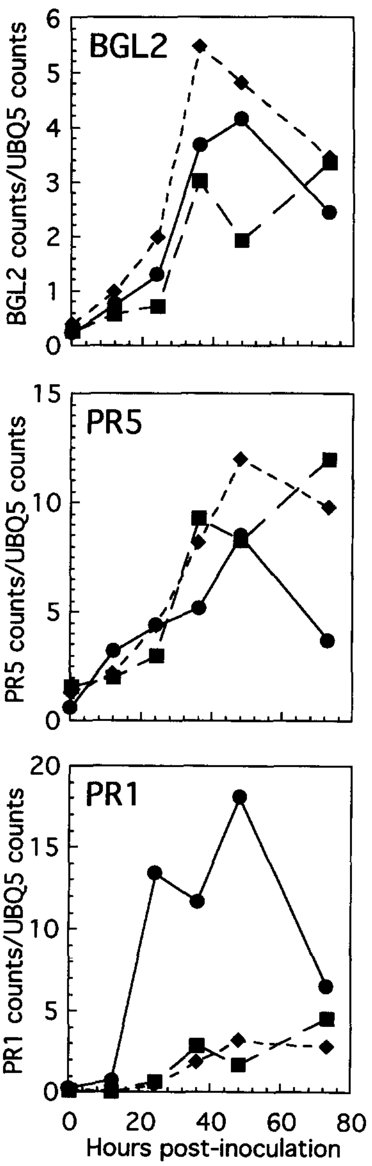

FIGURE 4.—mRNA levels of the defense-related genes *BGL2*, *PR5*, and *PR1* in wild-type, *npr1-2*, and *npr1-3* plants after infection with *Psm*ES4326 at a dose of 10^4 cells/cm^2. mRNA of the ubiquitin-encoding gene, *UBQ5*, was used as an internal standard. ●, wild-type Col-0; ◆, *npr1-2*; ■, *npr1-3*. The *npr1-2* and *npr1-3* plants used in this experiment were homozygous *npr1* F_3 families derived from a backcross to Col-0. In a previous experiment, which gave similar results, a different homozygous *npr1-2* F_3 family, and M4 *npr1-3* plants were used.

With the exception of *eds8-1*, none of the *eds* mutants displayed morphological alterations that cosegregated with the Eds$^-$ phenotype. The original M2 isolate of *eds8-1* was slightly compact and had serrated leaves. These phenotypes cosegregated with Eds$^-$ in the F_2 population described in Table 4.

DISCUSSION

We performed a direct screen for Arabidopsis mutants displaying an enhanced disease susceptibility phenotype. These *eds* mutants were found to represent 10 different genes, based on the results of complementation testing. They included mutants with defects in two different genes involved in phytoalexin biosynthesis, mutants with defects in the putative regulatory gene, *npr1*, and mutants with defects in seven other genes. Previous to this study, three or four genes (*pad1*, *pad2*, *npr1*, and *nim1*, with a possibility that *npr1* and *nim1* are allelic) that mutate to give an enhanced disease-susceptibility phenotype had been described (CAO *et al.* 1994; GLAZEBROOK and AUSUBEL 1994; DELANEY *et al.* 1995). The *pad* mutants were identified by screening for defects in phytoalexin accumulation following pathogen attack, the *npr1* mutant was found by screening for failure to induce a *BGL2-UIDA* fusion in response to salicylic acid, and the *nim1* mutant was found by screening for failure to develop resistance to *Peronospora parasitica* in response to treatment with INA (an inducer of SAR). Thus, all of these mutants were found by screening for defects in particular aspects of the defense response, whereas the *eds* mutants described here were identified in a direct screen for enhanced disease susceptibility.

Among the 12 mutations that we subjected to complementation testing, three were alleles of two previously known genes and the remaining nine defined eight different genes. The low frequency of allelic pairs among the *eds* mutations indicates that our screen was not saturating, so there is a high probability that there are more *EDS* genes remaining to be discovered. Since different plant pathogens vary with respect to their susceptibility to particular plant defense responses, it seems likely that sets of *eds* mutants could be identified in similar screens using different pathogens and that the various sets of such *eds* mutants would only partially overlap with each other and with the set reported here. The phenotypes of the *eds1* mutant, which is susceptible to several different avirulent *P. parasitica* strains that are recognized by different resistance genes, but is unaffected in interactions with other avirulent fungi and bacteria, support this idea (JANE PARKER, personal communication).

The *EDS* genes should include genes encoding components of signal transduction pathways involved in recognition of pathogen attack and activation of defense genes, as well as genes encoding proteins directly involved in defense responses that are important for limiting growth of *Psm*ES4326. The fact that we isolated mutants affected in 10 different *EDS* genes suggests that either there are a large number of regulatory factors involved in expression of a small number of crucial defense responses, or that a large number of defense responses are involved in limiting growth of *Psm*ES4326. If there are a large number of defense responses involved in limiting growth of *Psm*ES4326, it might seem surprising that mutations affecting particular responses have an easily observable effect on *Psm*ES4326 growth.

TABLE 3

Complementation testing of *eds* mutations

	Pollen recipient								
Pollen donor	Col-0	*eds-2*	*eds-4*	*eds-6*	*eds-8*	*eds-11*	*eds-13*	*eds-18*	*eds-48*
Col-0		+	*	*	*	*	*	*	*
eds-2	*		*	*	*	*	*	*	+
eds-4	+	+		*	*	*	+	+	+
eds-6	+	+	+		*	+	+		+
eds-8	+	+	+	−		*	+	+	+
eds-11	+	+	+	*	+		*	*	*
eds-13	+	+	*	*	*	+		+	*
eds-18	+	+	*		*	+	+		*
eds-48	+	*	+	*	*	+	+	+	

−, no complementation; +, complementation; and *, cross was not tested, but the reciprocal cross was tested.

However, there is mounting evidence that plant defense proteins can act synergistically to inhibit pathogen growth. Chitinases and glucanases, as well as a PR4-type protein and chitinase, act synergistically to inhibit fungal growth *in vitro* (Mauch *et al.* 1988; Melchers *et al.* 1994; Ponstein *et al.* 1994). Furthermore, chitinase and glucanase, as well as chitinase and a ribosome-inactivating protein, act synergistically to increase resistance to fungal pathogens when constitutively expressed in transgenic tobacco (Zhu *et al.* 1994; Jach *et al.* 1995). If such synergistic interactions are common, a mutation affecting any one of several different defense responses would be expected to cause an enhanced susceptibility phenotype.

In the original *npr1-1* mutant, the defense genes *PR1, BGL2,* and *PR5* were not induced in response to salicylic acid (Cao *et al.* 1994). Consistent with this result, we found that *PR1* mRNA levels in the *npr1-2 (eds-5)* and *npr1-3 (eds-53)* mutants were not significantly affected by salicylic acid treatment. When we examined the levels of *PR1, BGL2,* and *PR5* mRNAs in *npr1-2 and npr1-3* during pathogen attack, however, we found that induction of *BGL2* and *PR5* gene expression was not significantly affected by these *npr1* mutations, but that induction of *PR1* gene expression was greatly reduced. These data have two interesting implications. First, the observations that *npr1* mutations cause enhanced susceptibility to *Psm*ES4326 and a defect in *PR1* gene induction in response to *Psm*ES4326 infection suggest that *PR1* may play an important role in limiting growth of *Psm*ES4326 in infected leaves. This would complement the results of earlier work showing that constitutive *PR1* expression in transgenic tobacco leads to increased resistance to various fungal pathogens (Alexander *et al.* 1993). Second, although *PR1* mRNA levels in pathogen-infected *npr1* mutants was greatly reduced relative to that in wild-type plants, they were much higher than they were in uninfected plants. One explanation for this is that the *npr1* mutations are leaky; however, in the case of *PR1* induction in response to salicylic acid, no *PR1* induction was observed in either *npr1* mutant, which is inconsistent with the hypothesis that the mutations are leaky. Another explanation is that induction of *PR1* gene expression in response to *Psm*ES4326 infection results from the combined effects of at least two signaling pathways, one that is mediated by salicylic acid through *NPR1*, and one which is *NPR1*-independent. *BGL2* and *PR5* expression could be regulated similarly. If the contribution of the *NPR1*-independent signalling pathway(s) were large relative to that of the *NPR1*-depen-

TABLE 4

Segregation of Eds phenotypes in F_2 progeny from crosses between *eds* mutants and wild-type plants

		F_2		χ^2
Pollen donor	Recipient	Eds^+	Eds^-	(Eds^+:Eds^- = 3:1)
npr1-2	Col-0	42	13	0.05 ($0.8 < P < 0.9$)
npr1-3	Col-0	31	10	0.01 ($0.9 < P < 0.95$)
Col-0	*eds2-1*	51	10	2.41 ($0.1 < P < 0.2$)
eds3-1	La-*er*	150	51	0.02 ($0.8 < P < 0.9$)
eds4-1	Col-0	34	13	0.18 ($0.6 < P < 0.7$)
eds4-2	Col-0	39	15	0.22 ($0.6 < P < 0.7$)
eds5-1	Col-0	53	17	0.02 ($0.8 < P < 0.9$)
eds6-1	Col-0	53	18	0.005 ($0.9 < P < 0.95$)
eds8-1	Col-0	24	12	1.33 ($0.2 < P < 0.3$)

dent pathway, our finding that pathogen-induction of *BGL2* and *PR5* is not significantly affected in *npr1* mutants would be readily explained.

The results we have described demonstrate that plant defense responses can be dissected genetically, using direct screens for increased pathogen-sensitivity. The fact that some of the mutations were alleles of genes previously known to be involved in defense responses shows that the screen does yield bona fide defense response mutants. The mutations identified in this screen are unlike those obtained from screens for loss of resistance to pathogens carrying particular avirulence genes. Those screens yield mutations in cognate resistance genes and their associated signal transduction pathways, which result in qualitative conversion of the phenotype from resistant to susceptible (DONG *et al.* 1991; KUNKEL *et al.* 1993; YU *et al.* 1993; BISGROVE *et al.* 1994; CENTURY *et al.* 1995). In contrast, the *eds* screen yields mutations that have quantitative effects on the degree to which a virulent pathogen can grow and cause disease. Analysis of *eds* type mutants identified in this screen and similar screens that may be carried out using different pathogens should lead to new perspectives on the mechanisms by which organisms defend themselves from microbial attack.

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